Is Na+ channel blocker increase vincristine antitumor effect on neuroblastoma?

Antitumor effect of valproic acid and vincristine

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Abstract

Aim: The aim of the current study is the evaluation of valproic acid and Vincristine combination on neuroblastoma cancer cells and to answer the question if valproic acid (VPA) increase Vincristine (VCR) antitumor effect on the neuroblastoma cancer line or not. Material and Method: The neuroblastoma cell line was grown in culture medium. The different dose of VCR (0.5, 1 and 2 µg), VPA (5mM), VCR (0.5, 1 and 2 µg) + VPA (5 mM) was applied on neuroblastoma cancer cell lines for 24 hours. 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell viability, Anexin-V-FITC apoptosis, Total Antioxidant Capacity (TAC) and Total Oxidant Status (TOS) tests were done 24 hours after drug administration. Results: As a result of the tests, 2 µg VCR and VCR + VPA (2 µg + 5mM) reduced cell proliferation compared to the negative control group (P<0.05). Discussion: According to our result, valproic acid increased vincristine effect and reduced viability of cancer cells more effective than vincristine alone.

Keywords

Neuroblastoma; Valproic Acid; Vincristine; Annexin V; TAC

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Introduction

Neuroblastoma (NBL) is the most common solid tumor in children. NBL cells are undifferentiated cell type and spontaneously can develop benign ganglia or may turn to malignant type [1,2]. NBL incidence is 1 in 100,000 children especially in those under 5 years of age [3]. Forty percent of incidents are diagnosed in children younger than one year of age [4]. In most conditions, the tumor emerges in the retroperitoneal (75%), either in the adrenal gland (50%) or in the paraspinal ganglia (25%). The survival proportion of children with NBL is affected by the biology of the tumor, its place of origin, and the age of the illness [5,6]. Clinically, any effort to improve the treatment and diagnosis isvery important and it emerges to design new agents or adjuvant to reduce antitumor drugs dose [7].

Valproic acid ($C_8H_{16}O_2$) is commonly used in long-term epilepsy treatment in adults and children [8]. Previously it was proved that VPA increased cell death and phenotypic changes of neuroblastoma and SK-N-Be cells line. Valproic acid was used as an antitumor agent. Valproic acid (VPA) is one of the histone deacetylases (HDACs) inhibitors [9]. VPA induces tumor differentiation and apoptosis as well as suppressing tumor growth and metastatic processes [9,10]. However, the exact mechanism of VPA's anticancer effect is still uncertain [10]. Recently, the researcher has demonstrated VPA inhibits the invasiveness of bladder cancer but the same effect didn't see in prostate cancer cells [11].

Vincristine (VCR) is one of the vinca alkaloids, it acts as a mitotic inhibitor by binding to tubulin and stopping the polymerization, thus stopping the cell cycle in the metaphase of mitosis and killing cells [12]. VCR is widely used in the treatment of leukemia and solid tumors in childhood. Neurotoxicity is a common toxic display and limits the dose of a given drug [13]. Severe neurotoxicity may be due to a very small doses of VCR [14]. In childhood, due to the evolution of the body, tumor arises because of a reduced dose of anticancer drugs during adjuvant therapy.

In the current study, we used human neuroblastoma cells. For investigation of drugs effect and mechanism, we used MTT, TAC, TOS, and Annexin V-test at the end of 24 hours after the treatment. In our study, combination of VCR and VPA was done for the first time.

Material and Method

Chemicals and Reagents

VPA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). VCR was obtained from Sigma-Aldrich (Deisenhofen, Germany). The all chemicals, Dulbecco Modified Eagles Medium (DMEM), Fetal calf serum (FCS), neurobasal medium (NBM), phosphate buffer solution (PBS), antibiotic antimitotic solution (100×), L glutamine and trypsin–EDTA were obtained from Sigma Aldrich (St. Louis, MO, USA).

Cell Cultures

Neuroblastoma cells were obtained from the department of medical pharmacology department of Ataturk University (Erzurum, Turkey). Briefly, the cells after being centrifuged in 1200 rpm for 5 min were disseminated in 24-well plate by fresh medium (Neurobasal medium, with FBS 10%, B27 2%, and antibiotic 1%) and stored at incubator (5% CO_2, 95% moisture 37°C) [15,16].

Drug Administration

After gaining 85% confluency in 24-well plates the drugs were added. VPA (5mM) and VCR (0.5, 1 and 2 µg) were chosen to be added to the well plate and incubate for 24 hours (incubate in 5% CO_2, 95% moisture and 37 °C). For this aim, the experiments groups were divided into following 8 groups: Control group, VPA 5Mm, VCR 0.5µg, VCR 1µg, VCR 2µg, VPA 5mM + VCR 0.5µg, VPA 5mM + VCR 1µg and VPA 5mM + VCR 2µg.

MTT Assay

Twenty-four hours after exposure, the experiment was finished by adding 10 µL of MTT solution. Then the plates were incubated for 4 hours at 37 °C in a CO_2 incubator. DMSO solution (100 µL) was incorporated to all well to dissolve formazan crystals. The density of the Formazan crystals was read at a wavelength of 570 nm by the Multiskan ™ GO Microplate Spectrophotometer reader [17].

Total Oxidant Status (TOS)

The evaluation is made by calculating spectrophotometrically (Multiskan ™ GO Microplate Spectrophotometer reader). The intensity of the color linked to the quantity of oxidants status. The ingredients in the TOS kits were Reactive 1, Reactive 2, Standard 1, and Standard 2. In order to detect the TOS standard; 500 µl Reactive 1 was incorporated into the wells in which 75 µl plasma specimen was present and later reading the original absorbance value at 530 nm, 25 µl Reactive 2 was incorporated in the equal well and secondary absorbance was read at 530 nm at the end of the waiting duration of 10 minutes at room temperature. Standard 2 in the kit was used for Standard 2. By using the absorbance values acquired and the following formula, TOS standards were detected in H_2O_2 Equiv/mmolL-1 [16].

Total Antioxidant Capacity (TAC)

The ingredients of the kit were Reactive 1, Reactive 2, Standard 1, and Standard 2. In order to detect the TAC standard, 500 µl Reactive 1 was incorporated in the wells including 30 µl specimen and initial absorbance was read at 660 nm.Then, 75 µl Reactive 2 was incorporated into the equal wells and released to wait at room temperature for 10 minutes. At the end of the waiting duration, secondary absorbance value was read at 660 nm. While distilled water was used for Standard 1, Standard 2 in the kit was used for Standard 2. The absorbance values acquired were established according to the following formula and TAC standards were detected in Trolox Equiv/mmolL-1.

Morphologic Determination

The morphological determination was done by Leica microscope (USA). All the groups image was taken after 24 hours of exposure time (Figure1). For this aim, all images were taken at 20× magnitude. **Figure 1.**

Flow Cytometry Analysis

For evaluation of apoptosis pathway, Annexin V assay kit was used. The staining procedure was made according to manufacturer producer methods. The specimens were read by flow cytometry apparatus (CyFlow® Cube Flow Cytometer, Sysmex). The window x- axis showed annexin v- and y- axis showed propidium iodide value.

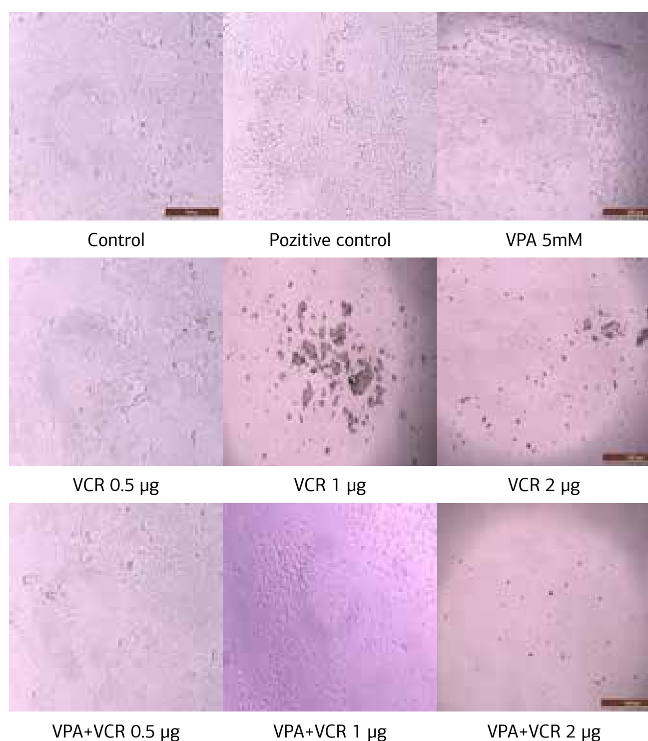

Figure 1. Microscopic view of each group after 24-hour treatment (20×)
Blue arrow: live cells, Red arrow: died cells, Triangle: Empty space

Statistically Analysis

The statistical analysis was done by one-way analysis of variance (ANOVA) and Tukey's HSD using the SPSS 22.0 software. $P<0.05$ was considered as a statistically important distinction for whole tests.

Results

In this study, VCR antitumor effect when combined with VPA was evaluated. These cells were exposed to VPA 5Mm, VCR 0.5µg, VCR 1µg, VCR 2µg, VPA 5mM + VCR 0.5µg, VPA 5mM + VCR 1µg and VPA 5mM + VCR 2µg for 24 hours. Only DMSO was added to the control positive group and the medium was added to the control negative group. MTT, TOS, TAC, and flow cytometry analysis tests were performed after exposure time.

MTT Assay

The survival rate of cancer cells after 24-hour drug exposure was calculated by using the MTT test (Figure 2). According to our results, pure VPA has the highest rate of viability compared to other groups. The lowest cell ability was seen in VPA + 2 µg VCR. When looking at the combination groups, the lowest viability rate was observed in the 2 µg VCR and the highest rate was observed in the 0.5 µg VCR; the VPA + 2 µg VCR group ($P<0.001$). In addition, 2µg VCR and 1µg VCR + VPA groups showed statistically difference in comparison to the control group ($P<0.05$).
Figure 2.

TOS

We evaluated the TOS test based on H2O2 equiv/mmol L-1 (Figure 3). The negative control and positive control groups TOS levels based on H2O2 equiv/mmol L-1were 2.3 and 2.6 respectively. In the combination groups, an increase in oxidant levels was seen in comparison to control groups. In addition, the oxidant level was higher in the 2µg VCR group than the VPA + 0.5µg VCR group. In the VPA + 2µg VCR and VPA + 1µg VCR groups statistically difference ($P<0.05$) observed. Furthermore, there was no statistically significant difference between the VPA, VCR (0.5, 1 and 2µg) and VPA + 0.5µg VCR groups compared to the control groups. **Figure 3.**

TAC

We evaluated the TAC test based on Trolox equiv/mmol L-1 (Fig. 4). The negative control and positive control groups TAC levels based on Trolox equiv/mmol L-1 were 5.6 and 5.8 respectively. A decrease in antioxidant levels was observed relative to the control groups in comparison to pure drugs (VPA 5.5, VCR (5.3, 5.1 and 4.8 respectively)). There was a decrease in the antioxidant levels of the combination groups (4.6, 4.1 and 3.8 respectively) relative to both control and pure groups. In the groups given VPA + 2µg VCR ($P<0.001$) and VPA + 1 µg VCR ($P<0.05$) according to the control group, statistically difference was observed.
Figure 4.

Flow Cytometry Analysis

We examined the apoptosis pathways in the neuroblastoma cell line after exposure to VCR, VPA and the combination of both drugs (Figure 5). The viable ratio of the negative control

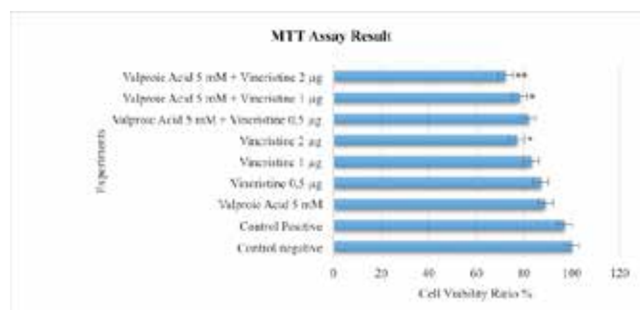

Figure 2. Viability rates for Neuroblastoma cells - MTT test chart
*P<0.05, **P< 0.001

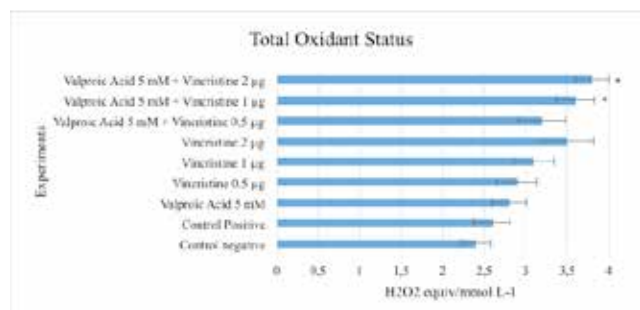

Figure 3. Total oxidant status test values read spectrophotometrically at 530 nm in cell culture fluid.
*P<0.05, **P< 0.001

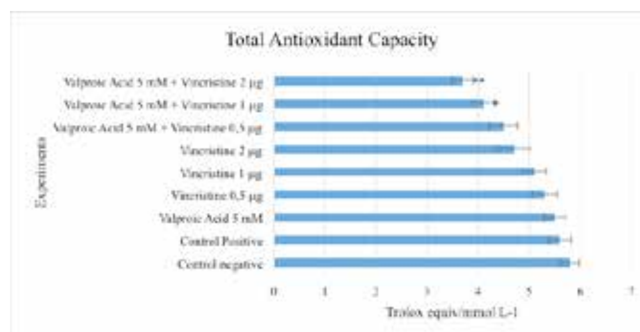

Figure 4. Total antioxidant capacity test values read spectrophotometrically at 660 nm in cell culture fluid.
*P<0.05, **P< 0.001

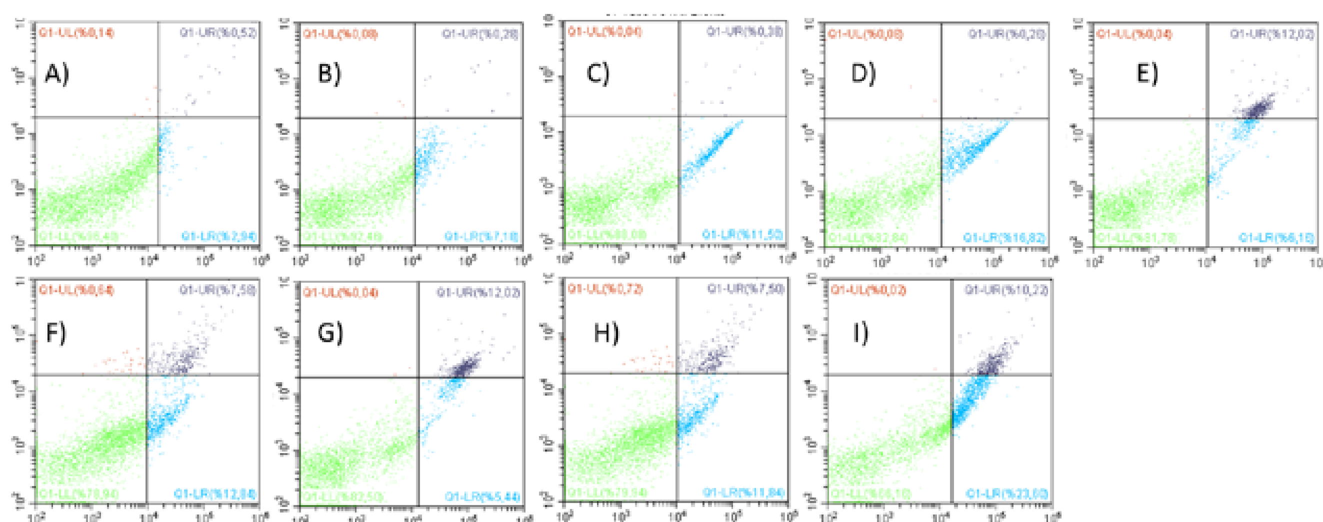

Figure 5. Neuroblastoma cells after treatment with VPA combined treatment for 24 hours.
*P<0.05, **P< 0.001

group was 99.4% and the positive control group was 92.46%. The positive control group has a higher necrosis value (7.18%) compared to the negative control group (2.94%). The pure VPA group viability level was 88.08%, necrosis 11.5%, late 0.38%, and early 0.04% apoptosis levels. In addition, the viability, necrosis, early and late apoptosis rate of the pure VCR groups (82.84%, 81.78%, and 78.94% respectively) shows decrease depending on the increasing doses. In addition, necrosis levels in combination group were 82.5%, 79.94% and 66.16% respectively. According to this data, pure 2µg VCR and VPA + 1µg VCR show the same viability ratio and nearly the same necrosis level. **Figure 5.**

Discussion

Neuroblastoma is biologically heterogeneous; the fallen-risk shape may retreat or grow automatically while the loud-risk shape expands relentlessly and can be quickly mortal [1,2,21,22]. NBL which are common in kids are very infrequent in grown-ups [1,2,7]. In grown-ups, it is hard to diagnose at a timely grade because the symptoms seldom show up until the illness has metastasized. Combining cure of DNA-detrimental drugs with VPA might also reduce many problems taken place during NBL cure of kids in clinical practice such as the reduction in drug dose and transient interruption of clinical cure induced by drug toxicity [7]. For this reason, a combination therapy with VCR and VPA may be hopeful in kids, but may not be influential in grown-up patients. In vitro, VPA has been indicated to inhibit proliferation in acute myeloid leukemia cells expressing P-glycoprotein and MDR- related protein 1 and to raise susceptibility to apoptosis in hepatoma cells resistant to epirubicin [23].

Priority of NBL, VPA has dramatically prohibited the interplay between endothelium and tumor cells. The attachment of single cancer cells to the vessel wall is a significant discovery in terms of promoting transendothelial migration and invasion into the enclosing tissue in the hematological invasion cascade. Thus, studies have shown that VPA may have a direct effect on the formation of metastases [24]. Tang R and Schuchmann M data have a correlation with our study, according to our data, VCR antitumor effect increased when VPA was added to culture. On the other hand, VPA increased VCR uptake by neuroblastoma cells [25].

Most neuroblastoma works are focused on moderate- and high-risk patients. A clinical work established that short- term and little dosage of integrated chemotherapy can cause an alike therapeutic influence, with a 3-year all survival ratio as high as 96 ± 1% and a 3-year eventless survival as high as 88 ± 2%, when compared with a long-term and high dosage chemotherapy exploiting anticancer agents [26]. Baker DL and colleague's data are near to us because the main purpose of the study is to try to decrease anticancer drugs dose. In the current study, our data showed that VPA can increase vincristine effect, in addition, VPA + VCR 1 µgr have shown the same effect with pure VCR 2 µgr [27].

Among high-risk pediatric patients, one more clinical trial indicated that jointed chemotherapy and stem cell transplantation can significantly increase the 3-year all survival ratio in contrast to chemotherapy alone. VCR is frequently used in chemotherapy to cure a pediatric patient with neuroblastoma background [28,29]. Because of the drug resistance, doses of VCR must be higher [23,30]. For this reason, Valproic acid is added to VCR for decreasing this dosage. VCR antitumor effect increased with the help of Valproic acid (Figure 2). VCR 2 µgr have an unacceptable effect on other body cells. For this reason, 1 µgr dose of VCR can induce cell cycle arrest at the M phase and induced apoptosis in neuroblastoma cells culture [31]. This data produces dependable indication for the practice of the VCR + VPA combination in neuroblastoma chemotherapy. In our study, the application of the VCR + VPA combination in neuroblastoma cells supports previous studies.

Conclusion

In conclusion, the VPA + VCR combination may inhibit the proliferation of neuroblastoma cancer cells. The VPA + VCR combination may be a new agent for the treatment of NBL as a single agent. Therefore, more work should be done to find a safe dose with the best effect of VPA and VCR.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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